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Efficacy of Bacteriophage against Uropathogenic *Escherichia coli* Isolated from Urinary Tract Infection

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ABSTRACT

This work involves the antimicrobial activities and effect of bacteriophage against Escherichia coli which is one of the major causative bacteria of urinary tract infection. Ten (10) urine samples were collected from patients after due consent and inoculated on Cysteine Lactose Electrolyte Deficient (CLED) agar. The isolates were characterized using standard microbiological procedures. It was further confirmed using the VITEK-2 Compact machine. The phage were isolated from sewage using deca-broth culture and incubated in shaker incubator for 48hours. Out of the ten (10) urine sample 60% (6/10) showed abnormal appearance and microscopic analysis respectively. Four of the ten (4/10) samples were isolated with Escherichia coli, followed by 10% (1/10) each of Proteus specie and Klebseilla specie. Escherichia coli concentration of 0.1ml was 75% and 100% susceptible to phage concentration of 4µl and 5µl respectively. This study has shown that bacteriophages can be effectively used as alternative therapy for treatment of urinary tract infection caused by uropathogenic Escherichia coli. This therapy will help to avert the adverse effect of antibiotics on adjunct organs which is part of complications during treatment with synthetic drugs. Hence, there is need for non-compounding process for the use of phages which are host specific without causing underlining damage to organs and tissues within the body.

Keywords: *Urinary Tract Infection, Bacteriophage, Escherichia coli, Susceptibility, Multidrug resistant.*

INTRODUCTION

Urinary tract infection is a common type of infection which involves the inflammation of the bladder, kidney or urethra. This infection usually affects either the upper or lower urinary tract thereby posing minor to major health challenges to patients. One of the foremost health challenges faced by humans and health providers is the bacterial resistance to antibiotics. Hence the recent moves by scientists towards identifying new antibiotics and as well as preserving existing ones, due to increasing level of microbial

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resistance to antibiotics and the need for alternative antimicrobials such as the introduction of bacteriophage (Owowo *et. al*, 2019). Urinary tract infections (UTIs) are one of the commonest kind of infections caused by bacteria with clinical cases amount to about 150 million annually. The disease affects the bladder, kidney and urethra i.e., the tube which links urine from the bladder to the outside (Johnson *et. al*., 2016).

Bacteria such as *Staphylococcus saprophyticus*, *Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumonia*, and *Proteus mirabilis* are among the causative bacteria associated with urinary tract infections. *Escherichia coli* happen to be the commonest bacteria responsible for causing urinary tract infection (Brooks, *et. al*., 2015). Uropathogenic *Escherichia coli* (UPEC) which is a strain of extra-intestinal pathogenic *Escherichia coli* are known to exist asymptomatically in the guts of humans for months or even years without causing any damages or harm (Kline *et. at.*, 2021). Uropathogenic *Escherichia coli* are known to be the major strain responsible for causing urinary tract infection especially in female. This is based on the direction of wiping their anus after defecation which may cause contamination of the urogenital orifice (Bach *et. al*., 2020).

Uropathogenic *Escherichia coli* (UPEC) are then introduced into the urethra during intercourse and can be introduced through anal to vaginal switching thus introducing the microbes to the system (Akter *et.al*. 2016). Antibiotics are usually the first line of therapy for urinary tract infection but extended- spectrum Beta Lactamase (ESBL) *Escherichia coli* strains known to be resistant to many drugs especially the penicillin family of antibiotics. Based on the recent increase in resistance of some bacteria strains to antibiotics especially due to certain factor and the quest for more efficient therapy, there is need to isolate bacteriophage associated with the bacteria and examine their efficacy on the isolate. So, this work is bent on accessing the efficacy of bacteriophage against *Escherichia coli* isolated from urinary tract infection sample using plague assay method.

MATERIALS AND METHOD

Study Area

The study was carried out in the Etalyx Medical Laboratory Services, a scientific research outfit in Oron Akwa Ibom State. Nigeria, but the scope is not limited to any geographical region as it covers more practical applications.

Sample Sampling

For purpose of the analysis, two type of sample were obtained which included sewage and urine samples. The sewage samples were collected from the works department of the university and abattoir within Oron. Urine specimens for the research were collected randomly from both gender of patient suspected to have symptoms of urinary tract infection from general hospital, Oron.

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Sewage Sampling

Under aseptic conditions, sewage samples were collected using sterile containers from the work unit of Akwa Ibom State University. The samples were collected by opening the tap of the sewage tanker of the works department. Sample from the abattoir were collected by scooping the upper layer of the sewage with a sterile spoon without allowing the spoon to touch the ground level. The sewage samples were transported to the Etalyx research laboratory for analysis.

Urine Sampling

Ten (10) mid-stream urine samples were collected with the assistance of a health provider from the intensive care unit with clinical symptoms of urinary tract infections. Samples were collected into a sterile open mouthed universal container from General hospital, Oron. Akwa Ibom State, after consents were taken from the patients. The samples were immediately transported to the Etalyx Scientific laboratory for further analysis.

Sample Analysis

Macroscopy**:** The appearance of the urine was observed and recorded as either cloudy, unpleasant smell, red and cloudy, clear, and amber. Urinalysis was carried out using combi – 10 immuno-absorbent reagent strips for detection of some biochemical parameters such as nitrate, protein, leukocyte which are known indications of urinary tract infection in urine. The urine was microscopically examined as wet preparation to detect for presence of pyuria (Significant value of white blood cells), red blood cells, cast, epithelial cells etc.
Microscopy**:** About 10 ml of the urine sample was aseptically transferred into a centrifuge tube. The samples were centrifuged at 2000 rpm for 5minutes. The supernatant fluid was poured off by completely inverting the tube into another container. A drop of the sediment was placed on a clean, grease free slide and covered with a cover-slip. The slide was examined microscopically using the 10X and 40X objectives to observe for presence of epithelial cells, casts, pus cells, crystals and other components in the urine sample. The remaining sediment were used for direct gram stain to observe for presence of gram negative and gram-positive cells.

Culturing of Samples using Filter Paper

Cystine lactose Electrolytes Deficient (CLED) agar was used to inoculate the urine sample after properly mixed by shaking it gently with the cap tightly screwed to avoid spillage. The filter paper was cut in rectangular forms and gently dipped into the urine sample aseptically. The paper was gently placed on the solidified agar plate under aseptic conditions. The procedure was repeated for all the urine samples and the plates were incubated aerobically at 37^0C for 24h.

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Inoculation by Streaking
The freshly collected urine specimen was properly mixed by shaking it gently with the cap tightly screwed to avoid spillage. Under aseptic condition, a sterile wire loop was dipped into the well mixed urine and inoculated directly upon the solidified plate of CLED agar. The plates were aerobically incubated at 37^0C for 24h.

Morphological Identification of Bacterial Isolates
Gram staining procedure described by Chesbrough (2010) was adopted to identify bacterial isolates morphologically.

Biochemical Identification of Isolates
Automated identification method using Vitek 2 compact system was used for biochemical identification of *Eschrichia coli.*

Isolation of *Escherichia coli* from sewage
Different samples of sewage were collected aseptically. The sewage samples were inoculated on Cystine Lactose Electrolyte Deficient (CLED) Agar plates without dilution. The analysis was given a clearer growth of the organism since it was qualitative analyses.

Phage Development
After isolation and characterization of the *Escherichia coli* from the samples, the isolated bacteria were used to develop phages from the same sewage sample. To develop the phages, enough bacteria isolate were made available to lyse, thereby multiplying the phages as well. By this, the *Escherichia coli* isolated from the sewage was cultured for 24 hours in a shakers incubator at 37^0C. Secondly, ten times the concentration of nutrient broth (Deca broth) was prepared and 5ml of the *Escherichia coli* culture was mixed with 5ml of the deca-broth and added to 45ml of raw sewage. This was referred to as the sewage culture. The sewage culture was incubated in the shakers incubator for 24 hours at 37^0C.

After the incubation, the sewage culture was centrifuged at 2500rpm for ten minutes to separate the lysed bacteria debris from the phage developed in the sewage culture. Due to the light weight of the phage particles, they were suspended in the supernatant of the centrifuged culture. This was carefully decanted into sterile container. To obtain a pure isolate of the developed phages, the supernatant was filtered through a 0.45μm membrane filter paper using a vacuum filtration system.

Plaque Assay
For the actualization of this final phase, the pure phage isolates, and *Escherichia coli* isolated from the UTI samples. First, Mueller Hinton agar plates were prepared while soft Mueller Hinton agar (0.7%) was kept in the oven at 45^0C to be used for making the

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monolayer. An aliquot of 0.1ml of each *Escherichia coli* isolate was mixed with dilutions 1µl, 2µl, 3µl, 4µl and 5µl of phage and added to 3ml of soft Muller Hinton agar in a test tube and poured on the surface of the solidified agar plate. The same was done with 10µl of phage. Also, as control, *Escherichia coli* isolate was mixed with 3ml of soft Mueller Hinton agar without adding phage and poured evenly on the surface of solidified nutrient agar. The assay was incubated in the incubator for 5 days and the plates were observed.

RESULTS

Morphological and Biochemical Identification of Isolates

Based on the clinical symptoms of the patients urine analysis was done indicating the parameters such as Protien, Blood, Bilurubin, Ntrite, Ketones, Ascorbic acid, Glucose and pH. Positive reaction of any of the parameters indicates case of abnormality in the patient. However, out of the ten urine samples, six (6) showed abnormality (table 1). Table 2 showed microscopic parameters such Pus cells (WBC), Red Blood Cell (RBC), Epithelial cells, Cast/Crystal, Yeast cells and Bacterial cells which showed some significant raised of Pus cells, Epithelial cells, Nitrite and bacterial cells indicating signs of urinary tract infections.

Table 3 shows the biochemical reactions and appearance of the various isolates from the ten urine samples analysed. Bacterial isolates number 1, 4, 6 and 10 showed positive reactions to the following biochemical reaction; Indole, Oxidase, ONPG, H2S and Gas production from oxidation of Glucose, indicating *Escherichia coli* as the causative agent of urinary tract infection. Isolates number 7 and 8 showed reactions to H2S and gas production and Indole, ONPG and Production of gas indicating *Proteus* specie and *Klebsiella* specie respectively. The isolates were all gram negative rods.

Table 1: Urine Analysis collected from symptomatic patients

No. of Sample	**appr.**	**Urine parameters**							**Remark**
		Prot.	Bil	Bld	Nitr.	Ket.	Glu	pH	Asc acid	
U1	Amb/Cldy Abnormal	++	-	-	+	-	-	5.6	-	
U2	Amb/Clr Normal	-	-	-	-	+	-	7.2	+	
U3	Turb/Cldy	+	-	-	+	-	-	6.0	-	Normal
U4	Pal/Cldy Abnormal	+	-	+	+	-	-	5.5	-	
U5	Amb/Clr Normal	+	-	-	+	-	-	6.5	-	

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U6	Amb/Clr Abnormal	-	-	++	-	-	-	6.0	-
U7	Pal/Cldy Abnormal	+	-	++	+	-	-	5.0	-
U8	Turb/Cldy Abnormal	++	-	+	+	-	-	6.0	+
U9	Amb/Clr Normal	+	-	-	-	-	-	6.5	-
U10	Amb/Clr Abnormal	+	-	++	+	-	+	8.0	-

Key: *U= Urine No=Number, appr=appearance, Prot=protein, Bil=Bilurubin, Bld=Blood, Nitr=Nitrate, Ket=Ketone, Glu=Glucose, Asc acid=Ascorbic acid, Amb=Amber, Cldy=Cloudy, Clr=Clear, Turb=Turbidity, Pal=Pale, (+)= Positive and(-) = Negative.*

Table 2: Microscopic Parameters from Symptomatic Patients

No. of sample	Urine parameters (PHF)						Remark
	WBC	RBC	Epith.	Cast/Cry	Yeast cells	Bact. cells	
U1	1-3	-	++	+	-	+	Abnormal
U2	0-2	-	+	-	-	-	Normal
U3	0-2	-	+	-	-	-	Normal
U4	2-3	-	++	++	-	+	Abnormal
U5	0-1	-	-	-	-	-	Normal
U6	2-3	-	++	++	++	-	Abnormal
U7	1-3	-	+	+	-	++	Abnormal
U8	1-3	+	++	-	-	++	Abnormal
U9	0-2	-	-	-	-	-	Normal
U10	2-4	-	++	+	-	++	Abnormal

Key: *No=Number, PHF=per high field, WBC=white blood cell, RBC=red blood cell, Epith.=Epithelial cell, Cry=Crystal, Bact=bacterial, (+)=positive and (-)=Negative*

Table 3: Bacterial Isolated from the urine of Symptomatic Patients

Sample	Urine pathogens						Remark
	Gram Rxn	Ind prodn	Oxidase	ONPG	H2S	Gas Prodn	
U1	G-ve	+ve	+ve	+ve	+ve.	+ve	*E. coli*
U2	-	-	-	-	-	-	No. path.
U3	-	-	-	-	-	-	No. path.
U4	G-ve	+ve	-	+ve	+ve	+ve	*E. coli*

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U5	-	-	-	-	-	-	No. path.
U6	G-ve	+ve	+ve	+ve	+ve	+ve	*E. coli*
U7	G-ve	-ve	-ve	-ve	+ve	+ve	*Proteus* spp
U8	G-ve	+ve	-ve	+ve	-ve	+ve	*Klebsiellas* spp
U9	-	-	-	-	-	-	No. path.
U10	G-ve	+ve	-	+ve	+ve	+ve	*E. coli*

***Key**: Rxn=reaction, Ind=indole, Prodn= Production, H2S=Hydrogen Sulphite, +ve=positive, -ve=Negative.*

In table 4, phage development and reaction with the bacterial isolates was observed by homogenizing the phage culture with bacterial isolates and incubated on the shaker incubator for 24h. The essence of the shaker incubator was for homogenous circulation of the nutrient and to give the bacteria stable growth rate. In bacteriophage assay, at the end os the three days, there was no zone of inhibition on the plate of Mueller Hinton agar and was re-incubated till fifth (5^{th}) to give enough growth time for the bacteriophage and their interaction with the *Escherichia coli*. At the end of five days incubation under 37^0C, it was seen to have discrete growth in form of dark patches and spots on the media. Thus the phage worked actively against the bacterial isolates see table 4.

Table 4: Antimicrobial Susceptibility pattern of Bacteriophage on *Escherichia coli* Isolates

Isolates	Phage Concentration					Positive	% Pos.
	1µl	2 µl	3 µl	4 µl	5 µl		
EC 1	R	R	I	S	S	2/5	40
EC 4	R	R	R	I	S	1/5	20
EC 6	R	R	R	S	S	2/5	40
EC 10	R	R	R	S	S	2/5	40

Key: EC = *Escherichia coli,* R = Resistant, S = Susceptible, % = percentage and Pos. = Positive

DISCUSSION

Urinary tract infection is one of the primary challenges faced worldwide due to increase in the laxity in various factors such as hygiene and use of antibiotics. Antibiotics are the first line of treatment for urinary tract infections, but some strains of the *Escherichia coli* called Extended-spectrum beta-lactamase *E. coli* have become more resistant to many of these

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drugs including ampicillin and tetracycline. The use of antibiotics for treatment has existed for decades hence the need for alternative therapy such as bacteriophage. The organisms obtained from the urine samples of those symptomatic patients with urinary tract infections were both gram negative which included *Escherichia coli, Proteus* specie, *Klebsiella* species. This is in agreement with Owowo *and* Udofia (2022) which stated that clinical routine trials have shown major causative pathogens of urinary tract infections are both gram positive and negative.

The selected isolates were sent in for antimicrobial susceptibility testing and automated identification using Vitek 2 compat machine which records about 96 – 97% precision of the organisms. The isolates were prepared under McFarland standard and infused into the machine for typical bio-pattern. The result obtained showed that the six (6) organisms isolated were *Escherichia coli* (4), *Proteus* specie (1) and *Klebsiella* specie (1), its conforms as described by Owowo *et. al*., (2019) that the above stated pathogens are associated with urinary tract infection. From bacteriophage culture development using raw sewage, it was deduced that the more the quantity of the test organism, the greater the chance of growing the phage in large numbers. The essence of increasing the quantity of the culture medium was to give a homogenous circulation of nutrient for the proliferation of the phage. The process connotes with the theory which describe phage as normal microbiota of the human bowel and other parts of the body.

The result of the phage bioassay involving interactions between varied quantity of sewage phages and *Escherichia coli* isolates on Muller Hinton agar showed phage can easily infect bacteria responsible for urinary tract infection. This is because at 0.1ml of *Escherichia coli* inoculums and 5µl of phage culture incubation for five days, there was distinct growth with some dark spots. At increased volume of both inoculums, there was clear growth with spots of inhibition zones. This conforms to the findings of Udom *et. al*., (2023) which describe the lysogenic and lytic interaction of the phage encounter with *Escherichia coli*.

CONCLUSION

It has been observed that binding–receptor of bacteriophage initiate the infection of their corresponding bacterial host and act as the primary determinant of host specificity. *Escherichia coli* which is one of the major causative pathogens for urinary tract infection will undergo a process till it get into full flesh detrimental ailment. The poor application of medication and wrong choice of drug can lead to severe damages due to changes in the pH state of the organs involved and mutation of the pathogens.

Bacteriophages are considered as an alternative treatment against multidrug-resistant bacteria such as *Escherichia coli*. This study has shown that bacteriophages can be effectively used as alternative therapy for treatment of urinary tract infection caused by uropathogenic *Escherichia coli*. This therapy will help to avert the adverse effect of

antibiotics on adjunct organs which is part of complications during treatment with synthetic drugs (Owowo *et. al*., 2019). Hence, there is need for non-compounding process for the use of phages which are host specific without causing underlining damage to organs and tissues within the body. Therefore, bacteriophages have shown a strong efficacy against uropathogenic *Escherichia coli* the major causative bacteria of urinary tract infection.

RECOMMENDATIONS

The zeal of this research is due to the frightening rate of urinary tract infection and reoccurrence treatment with even strong antibiotics, and the trending effect of excess synthetic antibiotics which have led to secondary necrosis of internal organs. Based on the need for alternative and reliable therapy for treatment of *E. coli* linked Urinary tract infection the following recommendations are made:

- More work on phage sequencing should be done
- Indiscriminate use of antibiotics should be discouraged
- Phage production for broad spectrum used should be recommended.

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